EXHIBIT D

ATTY. DKT. NO. 3[illegible]185.0010
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PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Leland SHAPIRO	Examiner: Jagoe, D.A.
Serial No.:	09/518,081	Art Unit: 1652
Filed:	January 27, 2000	
For:	METHODS AND COMPOSITIONS FOR INHIBITING APOPTOSIS USING SERINE PROTEASE INHIBITORS	

DECLARATION UNDER 37 C.F.R. §1.132

Commissioner for Patents
Washington, DC 20231
Sir:

I, Leland Shapiro, Assistant Professor at the University of Colorado, do hereby make the following declaration:

1. My current curriculum vitae is attached hereto as Appendix A;

2. I am named the sole inventor in the above-referenced application and I have read and studied the specification and pending claims of the above-referenced patent application; I have also studied the outstanding office action, which was mailed August 31, 2002 (Paper No. 13);

3. I understand from this office action that the Examiner has rejected certain of the pending claims based on an allegation of non-enablement of the claims as filed; I also understand that as part of the process of reconsidering rejected claims, the United States Patent and Trademark office takes into consideration evidence of enablement; what follows is a description in the prior art of the associated link between apoptosis and disease in general. Also described herein are the results of several *in vitro* and *in vivo*

studies involving administration to animals, in which apoptosis has been induced by administration of SU5416 (SUGEN compound, or 3-[2,4-dimethylpyrrol-5-yl)methylidenyl]-indolin 2-one), administration of Alpha-1-Antitrypsin, inhibits apoptosis unexpectedly and dramatically, as evidenced by significantly higher MLI interalveolar wall distance measurements, decreased evidence of emphysema and concomitant reduction of alveolar cell death. This is comparable to the loss of precapillary arterioles in the treated rats in the PCNA lung immunohistochemistry assay and decreased levels of terminal deoxynucloetidyl transferase dUTP nick end-labeling (TUNEL) of cells localized to the peribronchiolar, intra-alveolar, and septal cells, each of which in my opinion constitute surprising or unexpected results of the effectiveness of Alpha-1-Antitrypsin in inhibiting or reducing apoptosis;

4. In particular, with respect to demonstrating that there is indeed already established and well documented in the prior art a causal linkage between apoptosis and various diseases, I respectfully draw the Examiner's attention to the following publications which I believe clearly and unequivocally demonstrate the established mature of such linkage. The Publication of Hetts Steven W. (Hetts Steven W., "To Die or Not to Die", *JAMA*, January 28, 1998;**279:**4, a copy of which is attached herewith as Appendix B) is a review article that underscores the role of apoptosis in several diseases, including, for example, cancer, neurodegeneration, autoimmunity, heart disease,, and various other disorders;

5. Similarly, Grodzicky Tamara *et al.* (Grodzicky Tamara *et al.*, "Apoptosis in Rheumatic Disease", *The Amer. J. of Med.* January 2000;**108**, a copy of which is

attached herewith as Appendix C) is an article underscoring a role for apoptosis in the generation of rheumatic diseases, including, *inter alia*, rheumatoid arthritis, and systemic lupus erythematosis ("lupus"), systemic sclerosis, Sjogren's Syndrome, antiphospholipid syndrome, osteoporosis, osteoarthritis, and the seronegative spondyloarthropathies (including ankylosing spondylitis, psoriatuc arthritis);

6. The article of Honig Lawrence S. *et al.* (Honig Lawrence. S. *et al.* "Apoptosis and Neurologic Disease", *The Amer. J. of Med.*, March 2000;**108**, a copy of which is attached herewith as Appendix D) demonstrates that apoptosis likely plays a role in several neurodegenerative diseases. These include (but are not limited to) Parkinson's disease, Alzheimer's disease, Huntington' s disease, amyotrophic lateral sclerosis ("Lou Gehrig , s Disease"), and spinocerebellar ataxias.

7. The article of Mahidhara Raja *et al.* (Mahidhara Raja *et al.*, "Apoptosis in Sepsis", *Crit. Care Med.*, 2000;**28**:4 (Suppl.), a copy of which is attached herewith as Appendix E) demonstrates a role for apoptosis in the pathogenesis of sepsis. Therefore, the administration of anti-apoptosis inhibitors of host-derived serine proteases may be capable of treating sepsis;

8. The articles of Williams R. Sanders (Williams R. Sanders, "Clinical Implications of Basic Research", *The New England J. of Medicine"*, September 2, 1999: **341**, a copy of which is attached herewith as Appendix F) and James Thomas N. (James Thomas N., "Apoptosis in Cardiac Disease", *The Amer. J. Med.*, 1999;**107**:606-620, a copy of which is attached herewith as Appendix G) demonstrate that apoptosis likely has a role in several important cardiovascular diseases. These diseases include (but are not

restricted to) acute myocardial ischemia (angina pectoris), acute myocardial infarction ("heart attack"), thrombotic thrombocytopenic purpura (TTP), arrhythmogenic right ventricular dysplasia, long QT syndromes, and heart failure due to cardiomyopathy (including ischemic, dilated, infiltrative, and hypertrophic cardiomyopathies);

9. The article of Ueda Norishi *et al.* (Ueda Norishi *et al.*, "Apoptotic Mechanisms in Acute Renal Failure", *The Amer. J. of Med.*, April 1, 2000;**108**, a copy of which is attached herewith as Appendix H) demonstrates that apoptosis likely serves as a cause of acute renal failure, and that inhibitors of host-derived serine proteases may be used to treat acute renal failure. Specific diseases that can cause acute renal failure and that possess a component of apoptosis-induced damage include (but is not restricted to) renal failure due to hypoperfusion (ischemia of the kidney or pre-renal azotemia), renal artery stenosis, sepsis, acute renal failure due to contrast media exposure, endotoxin-induced renal failure, oxidant-stress-induced acute renal failure, and toxin or drug-induced acute renal failure (due to antibiotics, chemotherapy agents, immunosuppressive drugs, heavy metals, diuretic drugs). Also, apoptosis inhibition may be used as a mode of therapy to treat acute and chronic renal graft (transplant) rejection.

10. The article of Rust Christian *et al.* (Rust Christian *et al.* "Apoptosis and Liver Disease", *The Amer. J. of Med.*, May 2000:**108**, a copy of which is attached herewith as Appendix I) shows that apoptosis is likely involved in liver disease. Therefore, serine protease inhibition (using AAT or an AAT-like natural molecule or an AAT-like synthetic mimic) may be used to treat liver disease. Specific diseases include alcoholic hepatitis, drug or toxin-induced hepatitis, viral hepatitis (due to hepatitis A, B,

C, D, or E), and liver damage due to hypoperfusion ("shock liver", as occurs, for example, in the course of septic shock).

11. The articles of Aprikyan AG *et al.* (Aprikyan AG *et al.*, "Mutations in the Neutrophil Elastase Gene in Cyclic and Congenital Neutropnia", *Current Opinion Immunology*, 2001;**13**:535-538, a copy of which is attached herewith as Appendix J) and Ancliff Phil J. *et al.* (Ancliff Phil J. *et al.*, "Mutations in the ELA2 Gene Encoding Neutrophil Elastase are Present in Most Patients with Sporadic Severe Congenital Neutropenia But Only In Some Patients with the Familial Form of the Disease", *Blood*, November 1, 2001; **98:**9, a copy of which is attached herewith as Appendix K) are two papers that focus on two related diseases called cyclic neutropenia and congenital neutropenia. The articles demonstrate that two diseases manifest as severe reductions in blood neutrophil amounts, and this is caused by increased apoptosis of the blood neutrophils. This disease is associated with defects in neutrophil elastase (NE). NE is a host-derived serine protease. The mutations are gain of function variants of NE. Therefore, excessive activity of a host-derived serine protease (NE) can cause apoptosis. Thus, it is rational that inhibition of host-derived serine protease activity using a serine protease inhibitor (such as AAT, or an AAT-like natural molecule or an AAT-like synthetic mimic) reduces apoptosis;

12. The article of Bogdan Inja *et al.* (Bogdan Inja *et al.*, "Tumor Necrosis Factor-α Contributes to Apoptosis in Hippocampal Neurons during experimental group B Streptococcal Meningitis", *The J. of Infectious Diseases*, September 1997;**176**:693-7, a copy of which is attached herewith as Appendix L) demonstrates that serine protease

inhibition may be used to treat meningitis. As described, bacterial meningitis causes apoptosis of brain cells. Therefore, apoptosis inhibition using serine protease blockade is expected to reduced meningitis-associated brain cell death, resulting in clinical improvement. Thus, the article of Bogdan *et al.* demonstrates that serine protease inhibition may be used to treat meningitis. As described, bacterial meningitis causes apoptosis of brain cells. Therefore, apoptosis inhibition using serine protease blockade is expected to reduced meningitis-associated brain cell death, resulting in clinical improvement.

13. Finally, I respectfully draw the Examiner's attention to U.S. Patent No. 6,489,308, a copy of which is attached herewith as Appendix M, that clearly demonstrates the ability of Alpha-1-Antitrypsin or an AAT-like natural molecule or an AAT-like synthetic mimic to inhibit nitric oxide production coupled with the fact that nitric oxide is a well-established mediator of sepsis-induced organ dysfunction and of systemic hypotension. Thus, there are at least two mechanisms by which serine protease inhibition may treat sepsis; namely by inhibition of apoptosis and inhibition of nitric oxide production.

14. After having now demonstrated the clearly established link between apoptosis and disease in general, I now respectfully draw the Examiner's attention to the following *in vitro* and *in vivo* experiments described herein that demonstrate that the addition of Alpha-1-Antitrypsin was completely effective in blocking pharmacologically induced programmed cell death (or apoptosis induced in rats) by administration of SU5416 (SUGEN compound, or 3-[2,4-dimethylpyrrol-5-yl)methylidenyl]-indolin 2-one).

Apoptosis was induced in the rats by administration of SU5416; this is a standard art accepted method for studying apoptosis effects in rats as evidenced by the accompanying publication authored by Kasahara *et al.* (Kasahara *et al.* JCI Vol. 106:11 pp 1311-1319, a copy of which is attached herewith as Appendix N). Briefly, six-week-old Sprague-Dawley rats were divided into three groups: a. Control group (N=6); b. SU5416-treated group (N=6); c. SU5416 + AAT treated group (n=6). SU5416 was administered as a single injection of 20 mg/kg subcutaneously in a diluent (0.5% carboxymethylcellulose sodium, 0.9% sodium chloride, 0.4% polysorbate 80, 0.9% benzyl alcohol). AAT was administered as 30 mg/kg of AAT protein intravenously three times per week for three weeks. The AAT diluent was normal saline. Separate control experiments were performed using either injection of the SU5416 diluent, or injection of the AAT diluent. No differences in the data were observed in the animals receiving the diluents. Therefore, these results were pooled and used as the Control group for all data analyses;

15. Two histological-based assays and one terminal deoxynucleotidyl transferase-mediated (TUNEL) assay known to those of skill in the art as providing key indicators of apoptosis were employed, Alpha-1-Antitrypsin was completely effective in blocking pharmacologically induced programmed cell death (or apoptosis induced in rats). The first histological assay is the PCNA lung immunohistochemistry assay. The second histological assay is the Mean Linear Intercept (MLI) Assay. The third assay is the terminal deoxynucloetideyl transferase dUTP nick end-labeling (TUNEL) Assay.

Each of the three assays are disclosed in the article authored by Kasahara *et al.* (Appendix N);

16. In the PCNA lung immunohistochemistry assay, administration of SU5416 to rats preferentially induces apoptosis and causes the dissapearance of alveolar septal structures resulting in emphysema and concomitant alveolar cell death and is comparable to the loss of precapillary arterioles in the treated rats;

17. The MLI Assay is a measure of interalveolar wall distance. In the MLI Assay, administration of SU5416 to rats preferentially induces apoptosis and significantly increases interalveolar wall distances, indicating that the SU5416-treated lungs were emphysemous, i.e., there was a loss of alveolar septa, compared to animals not receiving the SU5416 drug. The process itself can be characterized by the absence of increased filtration by inflammatory cells as assessed by light microscopic examination of hematoxylin and eosin-stained slides and antimacrophage immunostaining, or fibrosis in SU5416-treated rat lungs compared to control lungs;

18. The TUNEL Assay technique is used to detect apoptosis and relies on labeling of DNA strand breaks in situ as evidenced by increased levels of terminal deoxynucloetideyl transferase dUTP nick end-labeling of cells localized to the peribronchiolar, intra-alveolar, and septal cells;

19. The results depicted in Table 1 (Appendix O), which presents the results of six separate experiments, unequivocally demonstrate that the addition of Alpha-1-Antitrypsin was effective in inhibiting or blocking pharmacologically induced programmed cell death (or apoptosis induced in rats) by administration of SU5416, as measured by the MLI Assay. Control animals receiving SU5416 alone showed no such inhibition of apoptosis as revealed by the significantly higher MLI interalveolar wall distance measurements (68.94 for SU5416 alone versus 57.51 for SU5416 plus Alpha-1-Antitrypsin). The data presented in Figure 1 (Appendix P) depict the graphic presentations of the results of this experiment. The inhibitory effect of Alpha-1-Antitrypsin as significantly inhibiting apoptosis is thus clearly evident;

20. The results depicted in Figure 2A (Appendix Q) unequivocally demonstrate that the addition of Alpha-1-Antitrypsin was effective in inhibiting or blocking pharmacologically induced programmed cell death (or apoptosis induced in rats) by administration of SU5416, as measured by the PCNA lung immunohistochemistry assay. Control animals receiving SU5416 alone showed no such inhibition of apoptosis as revealed by the emphysemous appearing-like structures evident in Figure 2B (Appendix Q). The inhibitory effect of Alpha-1-Antitrypsin as significantly inhibiting apoptosis is thus clearly evident;

21. Finally, the results depicted in Table 2 (Appendix R), which presents the results of four separate experiments, unequivocally demonstrate that the addition of Alpha-1-Antitrypsin was effective in inhibiting or blocking pharmacologically induced

programmed cell death (or apoptosis induced in rats) by administration of SU5416, as measured by the TUNEL Assay. Control animals receiving SU5416 alone showed no such inhibition of apoptosis as revealed by the significantly higher TUNEL values. The data presented in Figure 3 (Appendix S) depict the graphic presentations of the results of this experiment (greater than 14-fold or 43% for SU5416 alone versus approximately 3% for SU5416 plus Alpha-1-Antitrypsin). The inhibitory effect of Alpha-1-Antitrypsin as significantly inhibiting apoptosis is thus clearly evident;

22. In conclusion, these experiments show that addition of Alpha-1-Antitrypsin was effective in inhibiting or blocking pharmacologically induced programmed cell death or apoptosis. The administration to animals, in which apoptosis has been induced by administration of SU5416, of Alpha-1-Antitrypsin, inhibits apoptosis unexpectedly and dramatically, as evidenced by significantly higher MLI interalveolar wall distance measurements and blockade of apoptosis as detected using the TUNEL assay.

23. I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true, and further, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Dated: 1/29/03

By: Leland Shapiro
Leland Shapiro, M.D.

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